EXHIBIT B

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THE HEAT-SHOCK PROTEINS

S. Lindquist

Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

E. A. Craig

Department of Physiological Chemistry, The University of Wisconsin, Madison, Wisconsin 53706

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PERSPECTIVES

All organisms respond to heat by inducing the synthesis of a group of proteins called the heat-shock proteins or *hsps*. The response is the most highly conserved genetic system known, existing in every organism in which it has been sought, from archaebacteria to eubacteria, from plants to animals. Although certain features of the response vary from organism to organism, many are universal, or nearly so. All organisms examined produce proteins encoded by the hsp70 and hsp90 gene families in response to elevated temperatures. These proteins are among the most highly conserved proteins in existence. Also universally, several of the proteins induced by heat are induced by a variety of other stresses. Although the particular constellation of effective inducers varies somewhat from organism to organism, in nearly all cells anoxia, ethanol, and certain heavy metal ions induce the proteins. Furthermore, either the hsps themselves or their close relatives are present in all organisms at normal temperatures and play vital roles in normal cell function. This last finding has provided important information on the specific molecular functions of the proteins and will be discussed in detail in this review.

An early and long-standing assumption about the heat-shock response is that the hsps protect cells from the toxic effects of heat and other stresses; good evidence supports this view. There is, first of all, the very nature of the response. In all organisms, the induction of hsps is remarkably rapid and intense, in keeping with the notion that it is an emergency response. Moreover, there is a striking relationship between the induction temperature and the organism's environment. In different organisms the response is induced at very different temperatures. In each case, the organism would be expected to cope with such temperatures in its natural environment. Thus, in the fruit fly *Drosophila melanogaster*, induction occurs between 33–37°C, common temperatures on warm summer days (125). In thermophilic bacteria growing at 50°C, the proteins are induced when temperatures are raised to 60°C (51). In arctic fishes growing at 0°C, they are induced at 5–10°C (B. Maresca, personal communication). In mammals they are induced by fever temperatures (122), and in soybeans they are induced in the field on hot sunny days (104).

A particularly interesting example is provided by a variety of dimorphic pathogens that cycle between relatively cool temperatues in one phase of their life cycle and the warmer temperatures of their mammalian hosts in another phase. This change in temperature is accompanied by the strong induction of hsps, in both prokaryotic and eukaryotic pathogens. In what is almost certainly a related phenomenon, heat-shock proteins are immunodominant antigens in many of these infections. Both circulating antibodies and activated T cells have been shown to have specificity for the major heat-shock proteins

of organisms as diverse as *Mycobacterium leprae* (the causative agent in leprosy), *M. tuberculosis* (tuberculosis), *Coxiella burnetti* (Q fever), *Plasmodium falciparum* (malaria), *Schistosoma mansoni* (schistosomiasis), *Brugia malayii* (filariasis) (Selkirk et al), *Trypanosoma cruzi* (Chagas disease) and *Leishmania major* (Leishmaniasis) (reviewed in 247). In the case of *P. falciparum*, hsp70 was reported to be on the cell surface, but this has been disputed (6, 16). Given the fact that so many different hsps, not generally on the cell surface, have been reported as immunodominant antigens, the most likely explanation for their antigenicity is that they are extremely abundant proteins at high temperatures and are therefore processed by macrophages as major foreign antigens for presentation to lymphocytes. The induction of the heat-shock proteins might be further enhanced by the hostile environment in the macrophages which engulf them and which serve as host cells for some of the organisms.

The most compelling argument that hsps have a protective function is the strong correlation between their induction and the induction of thermotolerance. Thermotolerance experiments with similar design and general result have been performed in both cultured cells and a wide variety of organisms, including bacteria, yeasts, slime molds, soybean seedlings, fruit flies, and mice. The basic observation is that a group of cells or organisms is killed rapidly when shifted directly from its normal growing temperature to a much higher temperature, whereas a matched group, given a mild preheat treatment to induce hsps, is killed much more slowly. Moreover, preheat treatments induce tolerance to other forms of stress, and other forms of stress induce tolerance to heat. Apparently then, the heat shock proteins are induced by moderate stresses, stresses which themselves are not necessarily lethal, in order to protect the organism from even more severe stress. The system seems to make eminent biological sense.

Unfortunately, this simple picture dissolves on closer inspection. There are circumstances in which the induction of thermotolerance does not correlate with the induction of heat-shock proteins, and attempts to identify the role of any one protein in thermotolerance have met widely with frustration. Fortunately, much progress has come from unexpected quarters. The hsps themselves, or close relatives produced at normal temperatures, serve vital functions in normal cells. Their roles in normal cell function are providing important clues to their putative functions in thermotolerance. In this review we first describe what is known about individual proteins, concentrating on a few systems in which biochemical or genetic analyses have been particularly fruitful. We regret that the broad scope of the current heat-shock literature and the limits on the length of this review preclude the consideration of many interesting findings from other systems. We then examine the hypothesis that hsps play a vital role in thermotolerance.

HSP110

Most, but by no means all, eukaryotes produce proteins of greater than 100 kd in response to high temperatures. They have been characterized in detail only in mammalian cells. The 110 kd protein of murine cells is found in the nucleus, with concentration in the nucleolus of both control and heat-shocked cells (204). The protein separates from the phase-dense nucleolar body, forming a nucleolar cap, when cultures growing at normal temperatures become confluent or when actively growing cells are incubated without serum or are treated with actinomycin (195). Brief heat shocks do not lead to nucleolar segmentation in proliferating cells; in confluent cultures they reverse it. With longer heat shocks, hsp110 forms a ring-like structure at the nucleolar periphery (239). Immunoelectron microscopy indicates that hsp110 associates with the fibrillar component of nucleoli, the site of nucleolar chromatin (rDNA). Treatment of fixed cells with RNase eliminates staining (204), suggesting that the protein associates with RNA or with a complex of proteins that bind RNA. Since ribosome production is very sensitive to heat shock (155), it is speculated that hsp110 is induced to protect it. In this respect it is notable that a member of the mammalian hsp70 gene family also localizes to nucleoli and has been postulated to protect ribosome assembly (121, 166, 236). Unlike hsp110, this protein concentrates in the granular region of the nucleolus, the location of pre-ribosomes (238).

Other nucleolar proteins of 110 kd have been studied independently of the heat-shock response (52, 105). The peptide map of one, C23 (195), is similar but not identical to that of hsp110, and this protein, too, has been localized to the fibrillar region of the nucleolus (52). Unfortunately, the gene encoding hsp 110 has not been isolated, and no genetic analysis has been performed. Recently, the 104 kd hsp of *Saccharomyces cerevisiae* has been purified, used to produce antibodies, and shown to be a nuclear protein (K. Borkovich, S. Lindquist, manuscript in preparation). If it should prove to be an analog of the mammalian protein, this gene family will then be open to genetic analysis.

THE HSP90 FAMILY

Members of the *hsp90* gene family have been cloned and sequenced from several evolutionarily diverse organisms, including fruit flies, yeasts, chickens, mammals, trypanosomes, and bacteria. Sequence analysis of the cloned genes demonstrates that the proteins are very highly conserved. The proteins of even the most distantly related eukaryotes have 50% amino-acid identity, and all have greater than 40% identity with the *Escherichia coli* protein (12, 56, 65, 133). In all eukaryotes, a region of extremely high negative-charge density, which itself shows little sequence conservation, is located at the same relative position in the protein. The *E. coli* protein is missing this segment. All of the proteins, including that of *E. coli*, contain another, smaller region of high negative-charge density toward the carboxy terminus. The carboxy-terminal regions of these proteins are generally the most divergent, but the four most-terminal amino acids, *glu-glu-val-asp*, are the same in all eukaryotic hsp90s (in trypanosmes the second *glu* is replaced by *gln*; 56). It is remarkable that this sequence is also found at the carboxy-terminus of the eukaryotic hsp70 proteins. In other respects these proteins have little or no homology. The sequence must serve some important purpose, but what the purpose may be is presently unknown.

In virtually all cells, proteins of the hsp90 family are abundant at normal temperatures and are further induced by heat. In *D. melanogaster*, there appears to be only one gene in this family, *HSP83* (18). In addition to being constitutively expressed and induced by heat and other stresses, *HSP83* is developmentally induced during oogenesis (252). The haploid genome of the budding yeast *S. cerevisiae* has two genes in this family, encoding nearly identical proteins. One, *HSC83*, is constitutively expressed at a high level and is moderately heat-inducible; the other, *HSP83*, is constitutively synthesized at a lower level and is more strongly heat-inducible (K. Borkavich, F. Farrell, D. Finkelstein, S. Lindquist, in preparation). The more heat-inducible yeast protein is also developmentally regulated and accumulates as cells transit into stationary phase or begin to sporulate (110).

In vertebrate cells further diversification of the genes in this family has occurred with at least one encoding a signal sequence to transport the protein across the endoplasmic reticulum (109, 133, 143, 200). This finding may explain an earlier observation that antibodies against this protein stained the golgi (123). As is the case with the proteins of *Drosophila* and yeast, the other members of the vertebrate family appear to be abundant at normal temperatures, soluble, and predominantly cytoplasmic, with some relocalization in nuclei during heat shock (31, 42, 113, 218). A tumor-specific transplantation antigen, Meth A, has recently been identified as hsp90 (215). Although a small portion of the protein is found on the cell surface in this tumor line, it is postulated that it arrives there secondarily, by deposition of protein from lysed cells.

The ER protein is larger than the cytosolic protein, with an apparent Mwt on SDS gels of 94–108 kd versus 87–92 kd for the cytosolic form. It also contains the sequence *glu-glu-val-asp* at the same relative position in the protein, but in this case it is not C-terminal as the sequence extends an additional 24 amino acids (200). Again, the four most-terminal amino acids of the hsp90 ER proteins are identical to those of the hsp70 ER protein, in this case *lys-asp-glu-leu*. Here, the sequence has been shown to provide retention in the ER, preventing secretion, and is shared by other ER proteins (147). The

ER and cytosolic proteins are often not coordinately regulated. The ER protein is induced by glucose starvation (192) and has been named GRP94 (glucose-regulated protein), while the cytosolic version is induced by glucose restoration. The ER proteins are also induced by heat, steroids, and other agents, but their responses vary with cell type (68, 192, 194).

Biochemical Analysis

Biochemical analysis of hsp90, the cytosolic protein, in mammalian and chicken cells indicates that it associates with very different types of proteins but that, remarkably, it may serve a similar function in these different associations. The first protein with which hsp90 was shown to have a specific association was the transforming protein of Rous Sarcoma Virus, $pp60^{v\text{-}src}$ (26, 158). This tyrosine kinase associates with hsp90 and a 50 kd phosphoprotein immediately after it is synthesized. At or about the time it is released from association with hsp90, it is phosphorylated on tyrosine, inserted into the membrane, and activated as a kinase (27, 43). These results led to the proposal that hsp90 binds to the kinase, keeping it soluble and inactive, while it is transported to its proper location in the plasma membrane. (See Figure 1.)

Further evidence supports this hypothesis and suggests it is of more general significance. Five other transforming proteins with tyrosine kinase activity, *yes, fps, fes, fgr*, also form stable complexes with 90 and 50 kd proteins. In some cases this 90 kd protein has been identified as hsp90 (1, 128, 250). The kinases in these complexes are incapable of autophosphorylation (26, 251), a characteristic of the kinase monomer. Moreover, that fraction of the kinase that can be precipitated from cell lysates with anti-hsp90 sera is underphosphorylated. Finally, mutant $pp60^{v\text{-}src}$ proteins that are transformation defective form much more stable complexes with hsp90. If hsp 90 does have a general inactivating or transporting function, it might be expected to associate with the cellular equivalents of these tyrosine kinases, but this has not yet been described.

hsp90 does, however, associate with other cellular kinases. Highly purified preparations of the heme-controlled eIF2-α kinase contain hsp90 as a prominent component (179). In contrast with the tyrosine kinases, hsp90 appears to stimulate the kinase, thereby increasing phosphorylation of eIF2-α and inhibiting protein synthesis in reticulocyte lysates (D. W. Rose, B. Hardesty, W. J. Welch, manuscript submitted). Whether this contributes to translational regulation in heat-shocked cells is unclear. hsp90 also associates with casein kinase II and is a substrate for phosphorylation by that protein in vitro (179). Moreover, highly purified preparations of yeast protein kinase C contain hsp90 (F. O. Fields, J. Thorner, personal communication). Various members of the hsp90 protein family are phosphorylated in vivo in all organisms investigated, by an unknown mechanism of uncertain regulatory significance.

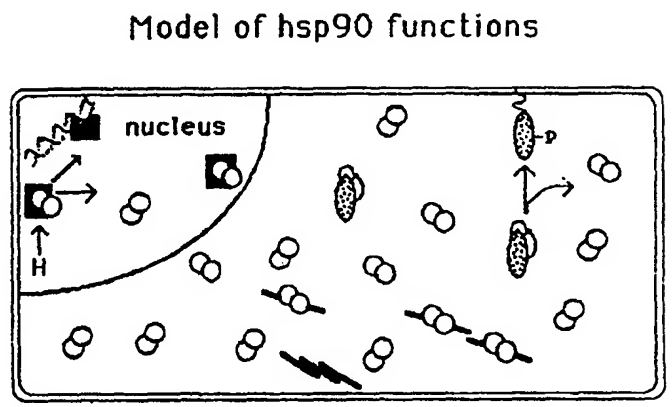

Figure 1 Model of hsp90 functions. The interaction of hsp90 with steroid hormone receptors (dissociated by hormone), tyrosine kinases (prior to insertion in the membrane), and other cellular proteins (including actin and tubulin). Hsp 90 is believed to exist as a dimer and is certainly in excess of the proteins with which it associates. At high temperatures, higher concentrations of hsp90 may be required to maintain the proteins in complexes.

Since hsp90 is not phosphorylated on tyrosine (26, 158), phosphorylation does not appear to be a consequence of its association with tyrosine kinases. That hsp90 is subject to phosphorylation by a DNA-dependent mechanism in vitro is intriguing but, at present, of uncertain import (231).

The other class of hsp90 associations studied in detail is the steroid-hormone receptor complex. All steroid hormone receptors studied to date, including the estrogen, progesterone and glucocorticoid receptors, can be isolated in the unactivated state (that is, in the absence of steroid hormones) as complexes with apparent molecular weights of ~300 kd and sedimentaion coefficients of 8–10S. These rather fragile complexes are dissociated by hormone, high salt (>0.25M KCl), or metal chelators, and are stabilized by low salt and molybdate ions. The complexes contain, in addition to the hormone-binding proteins, 90 kd proteins that have now been identified as hsp90 (33, 55, 98, 174, 175, 185). Since hsp90 is a very abundant protein and exists in vast excess to hormone-binding proteins, their association was initially suspect. However, the universality of the association, its specific stoichiometry (138, 174), and the fact that dissociation of hsp90 from the complex correlates with activation of the receptor for DNA binding (98, 175, 186) provide convincing evidence of its significance.

In the absence of hsp90, the hormone-binding receptor will bind to the DNA whether hormone is present or not (186). hsp90 binds neither DNA nor

hormone. Apparently, binding of hsp90 to the receptor prevents the receptor from binding to DNA until hormone disrupts association of hsp90 to the receptor. The specificity of the dissociated receptor for hormone-responsive DNA elements and its ability to activate transcription have not yet been examined. Although important questions about the role of hsp90 in regulating hormone-receptor activity remain unanswered, it has been postulated that the highly negatively charged domain in the amino-terminal portion of hsp90 forms an α-helix with a charge distribution that resembles the distribution of negatively charged phosphates in DNA. Hormone receptor would then bind to this region of hsp90 instead of binding to DNA, until hormone triggers its release (14, 17, 83). (See Figure 1). In support of this hypothesis, antibodies prepared against this charged domain of hsp90 immunoprecipitate most of the soluble hsp90 from cell extracts, but not the hsp90 that is complexed with steroid-receptor; this suggests that the charged domain is occluded by interaction with receptor (33). For the glucocorticoid receptor, evidence suggests association with hsp90 may inactivate the receptor by keeping it unfolded (256).

It is remarkable that a member of the hsp90 protein family induced by the steroid hormone antheridiol in the fungus *Achlya ambisexualis* is an integral part of the steroid-hormone receptor complex of this organism (28, 196). This suggests that the essential features of the hsp90-receptor interaction will be observed in all steroid-responsive organisms. It is interesting that the putative receptor-binding domain of hsp90 is missing from the hsp90 protein-analog of *E. coli* but is present in proteins of *S. cerevisiae*. There is no evidence that *S. cerevisiae* is naturally responsive to steroid hormones (although an estradiol binding activity has been reported; 29). Thus, if this protein domain should serve the same function in *S. cerevisiae* that it is proposed to serve in vertebrate cells, it may do so by interacting with other transcription factors. Steroid-hormone receptors belong to a large and ancient superfamily of transcription factors that are activated by structurally diverse ligands (63).

In broad outline, hsp90 appears to play a role in steroid receptor complexes similar to that in tyrosine kinase complexes, keeping the receptor inactive until the proper signal for activation is received. The very fact that it interacts with two such different proteins suggests it may interact with others in a similar manner. Recently, hsp90 has been reported to associate with actin in lymphocyte extracts, in a manner that is dependent on calcium and regulated by calmodulin (108, 154). Antibodies directed against hsp90 stain ruffling membranes in these cells, suggesting the interaction also occurs in vivo. It is postulated that the actin association provides a mechanism for transport of hsp90. In this regard, and considering the tendency of hsp90 to move into the nucleus with heat shock, it is intriguing that actin filaments rearrange during heat shock and may even be found in substantial quantities in the nuclei of heat-shocked cells (238). The protein also appears to associate with tubulin

both in vitro and in vivo (24). Given the high concentrations of actin, tubulin, and hsp90 in the cell, it seems likely that these associations are biologically significant.

Genetic Analysis

While biochemical characterization of the hsp90 protein family is more advanced in mammalian and chicken cells, genetic analysis is more advanced in yeast and *E. coli*. In both organisms, cloned genes can be used to construct deletion and disruption mutations in vitro, and these mutations can then be transformed into wild-type cells in a manner that converts the wild-type gene to the mutation. Experiments of this type demonstrate that the *HSP90* gene family is essential in *S. cerevisiae*. Individual mutations in either of the two closely related *HSP83* and *HSC83* genes are viable. Double mutations are lethal. The individual mutants have two interesting phenotypes. First, they do not grow at temperatures above 37°C; 39°C is the maximum growth temperature for the parental strain. (K. Borkovich, F. Farrell, D. Finekistein, S. Lindquist, manuscript in preparation). At 25°C both grow as well as the wild-type. Clearly, the proteins encoded by these two genes serve identical or nearly identical functions, but neither gene alone is adequate for growth at high temperatures. Thus, hsp90 is an essential protein, required in higher concentrations for growth at higher temperatures. This phenotype reflects the pattern of expression observed for this protein family in all organisms: the members of the hsp90 family are abundant, constitutively synthesized proteins that are also heat inducible. The phenotype of yeast mutants defective in *HSP83* and *HSC83* does not tell us whether the protein is required for a different purpose at higher temperatures or is required for the same purpose at all temperatures but in different concentrations. The latter fits more naturally with the proposed function of hsp90 in binding to other proteins and keeping them inactive until they have reached their proper location or until their activity is required. For such interactions to be biologically useful, they must be rather easily disrupted. High temperatures might simply drive the equilibrium more towards dissociation, requiring cells to produce a higher concentration of the protein to achieve the same level of complex formation.

The second phenotype associated with the individual mutations is a reduced ability to withstand exposure to extreme temperatures. They die more rapidly than do wild-type cells during exposure to 50°C. Notably, this difference in thermotolerance is observed in cells that have been grown in acetate, which forces them into respiratory metabolism, but not in cells that have been grown in glucose medium, which supports fermentative metabolism. It may be that hsp90 plays a role in thermotolerance, as classically defined, only in respiring cells. Alternatively, it may play an equally important role in fermenting cells,

but the concentration provided by a single gene may be sufficient for this purpose.

In contrast to the results in yeast, deletion of the *htpG* gene (which encodes the protein denoted C62.5, the only known member of this protein family in *E. coli*) is not lethal (12). However, like the individual deletions in yeast, the mutant does not grow as well as the wild type at high temperatures. The effect of the *htpG* mutation on survival at extreme temperatures has not been tested. The different effects produced by a complete loss of this protein in cells of *S. cerevisiae* and *E. coli* may be explained in several ways. First, another *E. coli* protein might cover the function of C62.5 in the deletion mutant. Arguing against this, no other genes cross-hybridize with the *htpG* gene, even at low stringency; no other proteins cross-react with a polyclonal antibody raised against the protein; and the *htpG* deletion strain does not overproduce any other protein that might thereby be suspected of compensating for its loss. A monoclonal antibody with specificity for mammalian hsp90 has been reported to cross-react with the *E. coli* lon protein (116), but by DNA sequence analysis, the proteins appear unrelated (A. Goldberg, personal communication).

A second possibility is that the protein performs a function that is essential in higher cells but not in bacteria. Simplistically, if the protein serves to ferry other proteins around the cell, as the biochemical evidence strongly suggests, the smaller size of bacterial cells might make this function valuable but not essential. Third, it is possible that the eukaryotic protein has acquired additional functions and that it is these newer functions that are essential. Again, the biochemical evidence is suggestive as the prokaryotic proteins lack the negatively charged region postulated to interact with steroid-hormone receptors.

In vertebrate cells, the association of hsp90 with steroid-hormone receptors and tyrosine kinases suggests it may serve as a type of "molecular chaperone," a function that, in the broadest sense, it may share with other heat-shock proteins (61). Unfortunately, this scheme rests almost entirely on in vitro analyses. Genetic analysis in yeast and *E. coli* has provided important general information but few specific biochemical hypotheses. The high degree of conservation in members of the hsp90 gene family and the similarity in their patterns of expression suggests that their roles are similar in all eukaryotes and, certainly in some respects, in bacteria. Given the many different proteins with which hsp90 is believed to associate, genetic analysis is likely to be complicated by pleiotropic effects. Almost certainly, a combination of biochemical and genetic investigations will be required to decipher all of the functions of hsp90. To such an end, a steroid hormone response has recently been produced in yeast cells by transforming them with the gene for the estrogen receptor together with a estrogen-responsive reporter gene (139).

THE HSP70 FAMILY

HSP70 encodes the abundant heat inducible 70 kd hsp. *HSP70* of most, if not all eucaryotes is a member of a multigene family whose genes are expressed under a variety of physiological conditions. The *HSP70*-related genes isolated thus far are related at the level of greater than 50% identity over their entire length. The evolutionary history of this multigene family is not well understood. However, in some cases (such as the *GRP78*-like genes) the similarities are greater among genes from different species than among genes of the same organism, indicating early gene duplication events and maintenance of the multigene family over time. A number of hsp70 and related proteins present in different cellular compartments and associated with a wide variety of cellular processes have been identified. Studies have revealed biochemical similarities among the related proteins from a single organism, as well as among proteins isolated from diverse organisms. All hsp70 and related proteins bind ATP with high affinity (36, 237, 255); many are very abundant in cells and often found in association with other proteins. Genetic analyses, which have been carried out only in *E. coli* and lower eucaryotes, show that *HSP70* and related genes are essential for growth either at high temperatures or at all temperatures, indicating a critical role in normal cellular physiology for the encoded proteins.

Drosophila melanogaster

During studies with *Drosophila melanogaster HSP70* was found to be a member of a multigene family (95, 230). The family includes 5–6 copies of *HSP70* and one copy of the heat inducible *HSP68* gene. In addition, seven other genes that are expressed during normal growth have been identified and denoted *HSC1–7*. *HSC1–6* have been mapped cytologically to 70C, 87D, 10E, 88E, 50E and 5C, respectively (44; K. Pulter, E. Craig unpublished results). Using monoclonal antibodies, the presence of two abundant proteins encoded by these genes, called hsc70 and hsc72, have been identified (111, 162). After heat shock of the whole organism, hsp70 and hsp68 become obvious spots when stained with Coomassie blue (162); however, they never attain a level higher than the normally present related proteins (162). hsc70, which is encoded by *HSC4*, is a cytoplasmic protein heavily concentrated around the nucleus. hsc72 is encoded by *HSC3*, and a number of results indicate that it is analogous to the mammalian glucose-regulated protein, grp78 present in the endoplasmic reticulum.

Cellular localization studies of hsps were first carried out in *D. melanogaster*. After heat shock, hsp70 was found to concentrate mainly within the nucleus and secondarily at cell membranes (223). This translocation is not dependent upon the temperature per se, because concentration in the nucleus is also observed after exposure to a hypoxic environment. During recovery

from heat shock hsp70 leaves the nucleus and is found mainly in the cytoplasm. hsc72 is also found in the nucleus after a heat shock (K. B. Palter, G. Gorbsky, G. Borisy and E. A. Craig, unpublished results).

Saccharomyces cerevisiae

The *S. cerevisiae* genome contains at least nine genes related to *HSP70* of higher eucaryotes. Eight of these genes, originally named *YG100-YG107*, have been renamed on the basis of structural and functional similarities: SSA1–4 (stress seventy family *A*; *YG100*, *YG102*, *YG106*, *YG107*, respectively); *SSB1* and *SSB2* (*YG101* and *YG103*, respectively); *SSC1* (*YG104*); and *SSD1* (*YG105*). Recently, another member of this family, the *KAR2* gene, has been identified (M. Rose, personal communication). The sequence relationships among these genes are complex (see Figure 2), with nucleotide identities ranging from 50–96%. The expression of the family members is modulated differently in response to changes in growth temperature. *SSA3* and *SSA4* are expressed at very low levels during steady-state growth at 23°C, but their expression is greatly enhanced upon upshift to 39°C (240). *SSA2* expression changes little upon shift to a higher or lower temperature. *SSB1* and *SSB2* transcripts are abundant during steady-state growth but rapidly

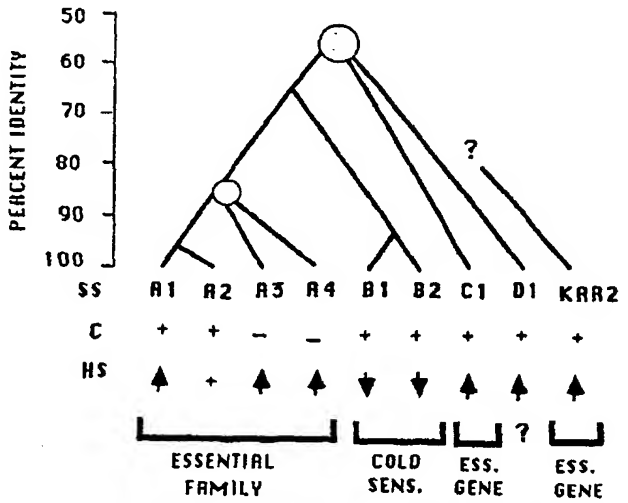

Figure 2 HSP70 multigene family of *S. cerevisiae*. Approximate percentage of nucleotide identities are based on partial or complete sequence data. The expression of the genes during exponential growth at 23°C (C) and after shift to 39°C (HS) and the placement of the genes into functional groups is compiled from Craig et al (47), Ellwood & Craig (62), Werner-Washburne et al (240), Jacobsen & Craig (45, 46), and M. Rose, personal communication.

decrease upon an upshift in temperature (46). *SSA1*, *SSC1*, *SSD1*, and *KAR2* transcripts are abundant during steady-state growth and increase 3–10 times upon an upshift in temperature (47, 62; Mark Rose, personal communication). Three members of the family have been precisely mapped. *SSA1* is located on the left arm of chromosome 1,7kb from the centromere (48); *SSC1* is on the right arm of chromosome X, 4–5 kb centromere proximal to *CYC1* (47); *KAR2* is on the left arm of chromosome X (M. Rose, personal communication).

Strains have been constructed containing mutations in members of the HSP70 family. Of the nine genes isolated, *SSD1* is the only member that has not been shown to be functional; no phenotype has been associated with the absence of the gene product. *SSC1* and *KAR2*, both of which are essential genes, are the only members whose absence has been found to result in a phenotypic effect in the absence of mutations in other genes. The *SSB* and *SSA* genes form two additional functional groups. Thus, at least four genetically identifiable functional groups comprise the *HSP70* gene family.

The most complex structural and functional subfamily includes *SSA1-4* (45, 240). Each of the protein products of these genes can substitute at least partially for the absence of the other three. *ssa1 ssa2* mutants are temperature sensitive for growth; they grow at 23°C but are unable to form colonies at 37°C. In addition, they are constitutively thermotolerant, able to survive short periods of time at high temperatures without a prior incubation at moderate temperatures. These cells behave as if they are permanently stressed, synthesizing at high level many heat shock proteins, including hsp90, hsp26 and Ssa4p. The reason for this production of stress proteins is not clear; either the cells are responding because they are "sick" due to the absence of Ssa1p and Ssa2p, or Ssa1p and Ssa2p are part of an important regulatory loop that is broken in their absence. Strains containing mutations in *SSA4*, *SSA3* or *SSA3* and *SSA4* were found to be indistinguishable from wild-type; they have the same growth and thermotolerance properties. Similarly, *ssa1 ssa2 ssa3* strains behaved like ssa1ssa2 strains. However, *ssa1 ssa2 ssa4* strains are not viable; spores of this genotype do not bud, and vegetatively growing cells containing an *SSA1* gene under the control of a conditional promoter undergo, on average, three cell divisions before growth stops after the termination of *SSA1* transcription. These results indicate that *SSA4*, which is constitutively expressed in the ssa1 ssa2 mutant, is allowing growth at low temperatures. Ssa3p is not synthesized in high amounts in the *ssa1 ssa2* mutant. To test whether *SSA3* could rescue the *ssa1 ssa2 ssa4* cells, *SSA3* protein coding region was put under the control of the constitutive *SSA2* promoter. This construction was able to rescue the growth of *ssa1ssa2ssa4* cells at 30°C, indicating that *SSA3* encodes a protein functionally similar to that encoded by *SSA1*, *SSA2* and *SSA4*. The reason for the inability of *SSA4* and *SSA3* to allow

growth at 37°C in the absence of *SSA1* and *SSA2* is not clear. Two-dimensional gel analysis of proteins indicate that Ssa4p or Ssa3p is present at levels similar to that of the Ssa1p and Ssa2p in wild-type strains or in strains containing the *SSA2* promoter-*SSA3* structural gene fusion. However, overproduction of Ssa3p or Ssa4p does allow some growth at 37°C. These results suggest that either *SSA3* and *SSA4* proteins are functionally different or predominately in a different cellular location from *SSA1* and *SSA2* proteins.

A strain carrying mutations in either *SSB1* or *SSB2* is indistinguishable from wild-type (46). However, a strain containing insertion mutations in both genes is relatively cold-sensitive for growth. It has an optimal growth temperature of 37°C, growing nearly as well as wild-type at that temperature but 2.4-fold slower at 19°C. A *SSA1* gene under the control of the *SSB1* promoter did not rescue the cold-sensitive phenotype of the *ssb1 ssb2* mutant. Conversely, the *SSB1* coding sequences placed under the control of the *SSA2* promoter could not rescue the temperature sensitivity of the *ssa1 ssa2* mutant. Therefore, the *SSA* and *SSB* genes must encode proteins that are either functionally distinct or present in different cellular locations such that they can not compensate for each other.

Analysis of the *SSA* mutants indicates that these proteins are involved with the posttranslational import of at least some proteins into the endoplasmic reticulum and into mitochondria (53). *ssa1 ssa2 ssa4* cells that contain *SSA1* coding sequences under the control of the *GAL1* promoter cease producing *SSA1* upon shift to glucose-based media. After several hours these cells begin to accumulate precursors to alpha factor (a secreted protein), carboxypeptidase Y (a vacuolar protein), and the beta subunit of F1 ATPase (a mitochondrial protein). Cell-free extracts prepared from *ssa1 ssa2 ssa4* cells after shift to glucose-based media are defective in the import of alpha factor precursor into the microsome vesicles. Furthermore, proteins purified from cells on the basis of their ability to facilitate import of alpha-factor in an in vitro system have been identified as *SSA1* and *SSA2* (38). Thus, both biochemical and genetic evidence indicate that *SSA* proteins are involved in the transport of proteins across membranes. It has been known for some time that an early step in the import of proteins into mitochondria is ATP dependent (59, 170), and evidence suggests that protein must be unfolded in order to pass through the membrane (225). No information exists at this time as to the actual role of SSA proteins in transport. *SSA* proteins may be acting in the cell as an "unfoldase," altering the conformation of proteins in an ATP-dependent manner that allows passage through membranes. Alternatively, they may be involved in some other aspect of the transport machinery and not interact directly with the translocated protein.

SSC1 is an essential gene. *ssc1* spores generated from a heterozygote germinate and undergo approximately three cell divisions before arrest (47).

The predicted amino acid sequence of *SSC1* has an additional 27 N-terminal amino acids when compared to *SSA1* protein. This proposed leader has structural features very similar to those found in proteins imported into mitochondria. The *SSC1* leader is rich in positively charged amino acids, devoid of acidic amino acids, and rich in the hydroxylated amino acids, serine and threonine. Furthermore, *SSC1* protein synthesized in vitro is imported into mitochondria and cleaved to a mature form in an in vitro assay (J. Kramer, M. Werner-Washburne, E. A. Craig, unpublished results). Presumably Ssc1p is a mitochondrial hsp70 protein.

Strains containing *KAR2* mutations were originally isolated because of a defect in nuclear fusion (173). Sequence analysis obtained from a complementing clone revealed a similarity to *HSP70* genes, especially *GRP78*, a mammalian member of the *HSP70* family found in the endoplasmic reticulum. The predicted *KAR2* protein has a leader sequence similar to those found in proteins imported into the endoplasmic reticulum. Furthermore, as expected of a homologue of GRP78, *KAR2* is induced by the glycosylation inhibitor tunicamycin. The *KAR2* gene is essential as shown by gene disruption experiments (M. Rose, personal communication). The reason for the defect in karogamy is not clear. The nuclear envelope may be dependent on proteins and protein complexes that enter and are assembled in the endoplasmic reticulum.

The genetic analysis in yeast leads to the conclusion that there are at least four genetically distinct groups of hsp70-related genes. This distinction could be due either to basic functional differences or to differences in cellular location. It is likely that both explanations are correct. Recent results indicate that at least two of the genes, *KAR2* and *SSC1*, produce products that are localized in the endoplasmic reticulum and mitochondria, respectively. Since both *SSC1* and *KAR2* are essential genes, the encoded proteins likely perform essential functions in these organelles. On the other hand, the genetic results with the *SSB* and *SSA* families indicate that there are functional differences amongst the proteins as well.

Escherichia coli

dnaK encodes a protein that is related to hsp70 of eucaryotes (11): DnaK is 50% identical in amino acid sequence to hsp70 of eucaryotes. There appears to be no other HSP70-related genes in the *E. coli* genome. *dnaK* was originally identified as a host gene necessary for lambda DNA replication (73, 206). Genetic data indicates that DnaK interacts with the P protein of lambda, since mutations in lambda phage that enable it to grow in a *dnaK*⁻ host map in the *P* gene. Biochemical experiments have confirmed and further defined its role in lambda DNA replication. Six proteins are required for the localized unwinding of duplex DNA at the origin of replication, prior to the binding of

DnaG primase: two lambda proteins—O and P—and four host proteins—DnaB (a helicase), DnaK, DnaJ and Ssb (single stranded binding protein). A complex of O, P, and DnaB form at the origin (117, 118, 246). The subsequent addition of DnaJ, DnaK, and Ssb proteins plus ATP results in an origin-specific unwinding of the DNA duplex. It is hypothesized that DnaJ and DnaK "loosen" the association between DnaB and P, so that DnaB is able to function as a helicase (246), thus permiting DnaG binding and subsequent DNA synthesis (see Figure A).

The *dnaK* gene was so named because *E. coli* DNA synthesis shuts off when mutant cells are shifted to high temperatures. Until recently, however, there was no direct evidence that DnaK was involved in host DNA replication. Sakakibara (184) isolated a new *dnaK* allele, *dnaK111* during a screen designed to isolate mutants defective in the initiation of DNA synthesis. It was known that mutations in *dnaA* (a gene required for DNA initiation) were suppressed in the presence of mutations in *rnh* which encodes RNAase H (93, 124). The suppression is thought to be due to the presence of some latent replication origins, which become active in the absence of RNAase H activity.

dnak111 was isolated as a temperature-sensitive mutant whose defects in

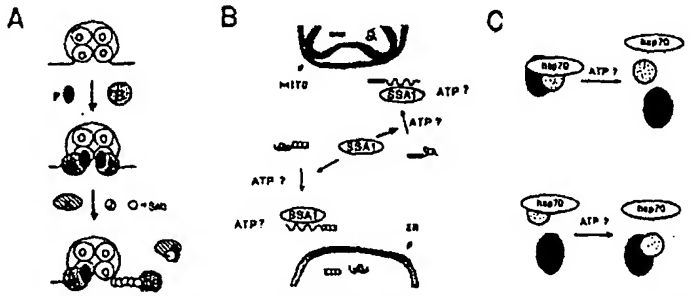

Figure 3 Schematic diagrams of hsp70 function. A. A proposed scheme for the role of DnaK and DnaJ in the initiation of phage lambda DNA replication. In step1, O protein binds to the lambda origin; in step2, P protein and DnaB protein bind; in step3, DnaK and DnaJ bind, disrupting the interaction between P and DnaB and thus allowing DnaB to catalyze the unwinding of the DNA and the subsequent binding of single stranded DNA binding protein Ssb. Adapted from Echols (58). B. Possible role of Ssa proteins in protein translocation across the endoplasmic reticulum (ER) and mitochondrial (MITO) membranes. The interaction with precursor proteins could either be direct as depicted or involve interactions with other components of the translocation machinery. Adapted from Deshaies et al (53). C. A general model of hsp70 and related protein function. hsp70 may be involved in disruption of already established protein-protein interactions between proteins, possibly in an ATP dependent manner (top), or in facilitating the establishment of "proper" interactions (bottom).

DNA synthesis could be relieved upon inactivation of the *rnh* gene. The *dnak111* mutant is unable to initiate a new round of DNA replication at high temperature after termination of the round in progress. DNA synthesis in both the *dnaK111* and *dnaA* mutants becomes temperature independent after reintroduction of a wild-type *rnh* gene. Unlike *dnaA* mutants, however, *dnaK111* mutants in the presence of an inactive *rnh* gene remain temperature sensitive for growth. The inability of *dnaI11* to grow at high temperatures even in the absence of *rnh* function suggests that this *dnaK* mutation causes pleiotropic effects. The defect in DNA synthesis can be corrected by inactivating *rnh* but other defects that cause cell death are not. These results imply that DnaK carries out multiple important functions in the cell.

Other genetic analyses have shown that *dnaK* is essential at high temperatures, and perhaps at lower temperatures as well. Cells containing deletions of *dnaK* can not grow at 42°C (161; P. Kang, E. A. Craig, unpublished results). The results at lower temperatures are more equivocal, because it appears that *dnaK* null mutants quickly acquire secondary mutations that allow more vigorous growth (P. Kang, E. A. Craig, unpublished results; A. Bukau, G. Walker, personal communication). Original transductants are extremely filamentous; cells containing secondary mutations are less so. Since cells that do not undergo this change are incapable of continued growth at 30°C or grow very poorly, it is suggested that *dnaK* is necessary for growth at temperatures other than 42°C.

The filamentation caused by *dnaK* null alleles is suppressed by plasmids that carry the *ftsZAQ* genes, which are necessary for normal cell division (A. Bukau, G. Walker, personal communication). The notion that *dnaK* may be involved in cell division, either directly or indirectly, is also supported by the finding that *fam-715* and *rpoH* genes are allelic (214). *fam* has been identified as a gene affecting cell division; *rpoH* encodes the heat-shock specific sigma factor and thus regulates the transcription of *dnaK* and other heat-shock genes.

It is not clear why strains containing *dnaK* null alleles die. Although DnaK is involved in the initiation of DNA replication, its role in the process does not seem to be essential. However, second site mutations that allow growth of *dnaK* null alleles have been isolated (161; P. Kang, E. A. Craig, unpublished results). In addition, overexpression of a wild-type gene allows cell growth at 41°C. This gene, which appears to be previously unidentified, maps to 4 minutes on the *E. coli* chromosome (P. Kang, E. Craig, unpublished results). Analysis of such suppressors may lead to an understanding of the essential functions of *dnaK*.

Dnak has been purified to near homogeniety, and the purified protein has been found to bind tightly to ATP and to have a weak DNA-independent ATPase activity (254, 255). Only 15–20 nmol of ATP are hydrolyzed to

ADP and Pi per milligram of protein per minute at 30°C, representing a turnover number of only one ATP molecule per minute. The DNA-independent nature of the ATPase activity was somewhat unexpected, since a number of proteins involved in DNA replication which also possess ATPase activity show a DNA dependence of the reaction, (e.g. as DnaB, protein m, helicase 1, and helicase 2). The purified protein is capable of self-phosphorylation on a threonine residue. In vivo about 5% of the total cellular DnaK is present in the phosphorylated form after labeling with ^{32}P. The DnaK protein also possesses a 5'-nucleotidase activity which is inhibited by AppppA (20). In vivo analysis of temperature sensitive *dnaK* mutants revealed that five proteins, normally phosphorylated, do not become so at the nonpermissive temperature. Two of the proteins, phosphorylated on threonine residues, have been identified as glutamine tRNA synthetase and threonyl tRNA synthetase (229).

Mammalian Cells

Four members of the human hsp70 protein family have been identified. These proteins have been called by various names in the literature (see Table 1). Here we refer to the human proteins as hsp70, hsp72, p72 and grp78, the nomenclature used by B. Watowich & R. Morimoto (234). hsp70, the major heat-inducible protein (236) is also a cell cycle regulated protein (140). Furthermore, it is under the control of adenovirus E1A protein and is often referred to as the 72k heat-shock protein. hsp72 is a protein which is expressed only after heat shock. p72 is expressed at high levels in growing cells

Table 1

Protein[1] name	Other name(s)	pI	Regulation
hsp70	72K[2]; hsx70[3]; SP71[4]; hsp68[5]	5.8-6.3	Major heat-inducible 70K; basal expression; serum stimulated; cell cycle regulated; E1a inducible
hsp72	hsp70	5.6-5.8	No basal expression; heat inducible
p72[6]	73K[2]; hsc70[5] hsc73[3]	5.5-5.6	High basal expression; slightly heat inducible
grp78	BIP; hsp80	5.2-5.3	High basal expression (especially in secretory cells; expression enhanced by glucose deprivation, calcium ionophores, glycosolation inhibitors, etc.

1. Using the nomenclature of Watowich & Morimoto (234) 2. Welch & Feramisco (236); 3. Pelham (165); 4. White & Currie (241); 5. Lowe & Moran (130); 6. a major heat-inducible protein in primates, but not found in rodents.

and is often referred to as the 73k heat-shock protein. grp78 is a glucose regulated protein located in the endoplasmic reticulum (146). Rodent cell lines synthesize three members of the hsp70 family; there appears to be no direct equivalent of p72 (that is, a protein present in cells at a high basal level and also induced dramatically upon heat shock). A glucose regulated protein, grp75, present in mitochondria appears to be hsp70-related (W. Welch, personal communication). The total number of *HSP70* related genes (proteins) in mammalian cells is not clear. Most of the analyses have been carried out in tissue culture cells. Transcripts from two previously unidentified *HSP70* related genes have been found in mouse spermatogenic cells (2, 248, 249). After careful examination of tissues during development more members may be found. At least 10 *HSP70* related genes have been found in the human genome, but the number that are functional genes is not clear (144). Human *HSP70* related genes have been shown to be located on chromosomes 6, 14, 21, and at least 1 other chromosome (79, 88). As discussed below, biochemical analysis of the mammalian hsp70 related proteins has provided much information.

CLATHRIN UNCOATING ATPase Clathrin uncoating ATPase was identified as a member of the hsp70 family based on the copurification of the uncoating ATPase and p72, two-dimensional gel analysis and immunological cross-reactivity (36, 216). The uncoating ATPase was purified on the basis of its ability to release clathrin triskelions from bovine brain coated vesicles (187). Coated vesicles mediate selective intracellular membrane transport. Clathrin, which is the major structural component of the coated vesicle basket, is found as a three-legged structure called a triskelion. Clathrin triskelions can spontaneously self-assemble into "cages" which resemble the coats of coated vesicles. The uncoating enzyme hydrolyzes ATP in a clathrin-dependent manner, driving cage disassembly (23). During an uncoating reaction, clathrin triskelions are released intact with the uncoating protein in a stoichiometric complex; 3–4 molecules of ATP are hydrolyzed per triskelion released. A two-stage model for the mechanism of clathrin cage disassembly has been presented (189). First, the energy of ATP hydrolysis drives the transient displacement of a portion of a triskelion from the cage. This transient displacement is thought to reveal a previously buried site, which is then bound to the uncoating protein, thus stabilizing the displacement. The triskelion-uncoating complex is released when all points of attachment of the cage are broken (190). In the in vitro assays, the uncoating protein bound to triskelions can be recycled. Since the apparent affinity of uncoating protein for cages is five times higher than that for unassembled triskelions, recycling is a spontaneous process. However, when the reaction is carried out with coated vesicles isolated from cells, the reaction is stoichiometric, that is, a 70K

protein is involved in only one round of uncoating (81). Free clathrin does not inhibit the reaction (81). The uncoating ATPase is not permanently inactivated. If separated from clathrin, it is capable of participating in uncoating again.

At the present time, however, there is no evidence that p72 functions as an uncoating ATPase in vivo. It is unlikely that clathrin uncoating is the only function of p72. In some tissues there is a 30-fold molar excess of p72 over clathrin (76). Mouse stem cells, for example, express p72 at very high levels, but clathrin is barely detectable.

P72 AND HSP70: BINDING TO NUCLEAR MATRIX AND NUCLEOLI Upon heat shock, both p72 and hsp70 migrate to the nucleus and are associated with the "insoluble matrix" in a salt resistant manner (238). The proteins subsequently become associated with nucleoli. Nucleoli are particularly sensitive to hyperthermia; severe structural changes which persist for several hours after a heat shock can be observed microscopically. The recovery of nucleolar morphology occurs more rapidly in cells that are constitutively overexpressing Drosophila hsp70 due to transformation with the Drosophila *HSP70* gene on a plasmid (165). The interaction of p72 and hsp70 prior to heat shock appears to be weak and readily reversible since they are released from nuclei upon lysis of cells with isotonic buffer. After a heat shock, the association becomes strong; however, they are rapidly released in vitro in the presence of ATP (121). Nonhydrolyzable ATP analogues are not effective in affecting release.

INTERACTION OF P72 AND HSP70 WITH CELLULAR TUMOR ANTIGENS Cells transfected with the gene encoding p53, a cellular oncogene, and an activated *ras* oncogene become morphologically transformed. In such cells, p53 is more abundant and stable than in normal cells, and it is associated with p72 (92) and with a lower affinity, with hsp70 as well (171). Mutations in the gene encoding p53 that activate its transforming potential also result in the synthesis of mutant proteins which show preferential association with p72 and have an increased half-life (70). It has been hypothesized that p53 is stabilized because of its interactions with p72. This hypothesis is based on the precedent that the apparent stability of p53 in SV40 transformed cells is caused by its association with the large T antigen. The p72-p53 complex can be dissociated in vitro with ATP, but not with unhydrolyzable analogues. Interestingly, p53 synthesized in *E. coli* is found in association with DnaK (40).

In addition, a mutant medium T antigen encoded by a nontransforming mutant of polyomavirus is reportedly associated with p72, whereas medium T antigens of wild type and at least one transformation-competent mutant polyomavirus are not (232).

GRP78 The grp78 protein, a member of the hsp70 family (146), was originally identified as a protein whose rate of synthesis increased when cells were starved for glucose (119, 193), and it was later shown to be induced under a variety of other conditions including anoxia, paramyxovirus infection and treatment of cells with glycosylation inhibitors or calcium ionophores, but not by heat shock. An inverse correlation between the rate of glycosylation and the steady-state level of the GRP78 transcripts has been observed (35). grp78 has a hydrophobic amino terminal leader sequence which by analysis of gene fusions has been shown to be competent for transport into the endoplasmic reticulum. grp78 is very similar or identical to BiP (146), a protein originally reported to associate with immunoglobulin heavy chains in preB cells that do not make light chains. In normal B cells and plasma cells, a smaller fraction of the intracellular heavy chain is also associated with BiP (87). BiP can be released from BiP-immunoglobulin complexes in vitro by incubation with ATP (146). grp78 (BiP) is present in the lumen of the endoplasmic reticulum of a large number of different types of mammalian cells. It binds transiently to a variety of wild-type secretory and transmembrane proteins and permanently to proteins that are unfolded or misfolded. For example, mature hemagglutinin of influenza virus is a trimeric glycoprotein. Mutants of hemagglutinin that fail to be transported from endoplasmic reticulum to the Golgi apparatus are not efficiently assembled into trimeric structures and remain in a partially unfolded state, associated with grp 78 (75). Mammalian cell lines which have decreased amounts of grp78 expression have been constructed using antisense RNA (54). These lines show increased secretion of mutant proteins.

It is interesting that a 30 amino acid peptide isolated from rat cells with complete identity with the carboxyl terminus of grp78 has been identified as a steroidogenesis-activator polypeptide (164). This peptide, whose activity is increased in the presence of cyclic AMP, is postulated to regulate a commitment step in steroid formation that is under hormonal control, the conversion of cholesterol to pregnenolone. It is thought that this peptide is derived from a larger precursor, presumably grp78 or a closely related protein.

OTHER CHARACTERISTICS OF HSP70 AND RELATED PROTEINS Rat p72 and hsp70 are associated with nonsterified fatty acids, palmitic acid, stearic acid and a small amount of myristic acid (86). The role of fatty acids in hsp70 function is not clear. A number of covalent modifications of hsp70 and related proteins have been reported in the literature. Some of the mammalian proteins are methylated at lysine and arginine residues (233). Dictyostelium hsp70 has been reported to be phosphorylated (129). Attempts to detect phosphorylation of the chicken, mammalian, and Drosophila proteins have not been successful (85; S. Lindquist, K. Palter, E. Craig, unpublished results), however,

phosphorylation of hsc72 of Drosophila has been detected (S. Lindquist, K. Palter, E. Craig, unpublished results)

Discussion of the Hsp70 Function

From the results presented in the previous sections it is obvious that HSP70 and related genes have been implicated in a variety of cellular processes. At first glance these processes—which include DNA replication, transport of proteins across membranes, binding of proteins in the endoplasmic reticulum, and uncoating coated vesicles—appear to have little in common. However, they may all involve the disruption of either intramolecular or intermolecular protein-protein interactions. The model for uncoating of coated vesicles involves the disruption of the coated vesicle basket by disruption of the interactions between the clathrin triskelions in an ATP dependent manner. The hypothesized role of DnaK in lambda replication is the disruption of the interactions of DnaB and P proteins such that DnaB is able to function as a helicase. It has been hypothesized that the role of the SSA yeast proteins in protein import is to disrupt intramolecular interactions of proteins such that they can attain an import-competent conformation. Varshavsky and colleagues (71) suggested that heat-shock proteins might bind to denatured or abnormal proteins after a heat shock to prevent their aggregation and thus to prevent cellular damage. Pelham (166) extended this hypothesis to include the assembly and disassembly of proteins and protein-containing complexes both during normal growth and after a heat shock. As noted above, hsp70 and related proteins all have a high infinity of ATP. Most of the "reactions" described involving 70K proteins, including the release from nucleoli require, or are at least thought to require, the hydrolysis ATP. Probably the disruption of the protein-protein interactions require the energy generated from ATP hydrolysis. The findings that hsp70-related proteins reside in the endoplasmic reticulum and mitochondrium and that hsp70 is translocated into the nucleus upon heat shock suggest that 70K proteins perform important functions in all cellular compartments.

GRO E-HSP58

The *groE* genes of *E. coli* were originally identified as genes necessary for productive growth of bacteriophage lambda and T4 (74). The two genes *groEL* and *groES* comprise an operon under heat shock control (212) located at 93.5 minutes on the *E. coli* chromosome (84). They encode abundant proteins; *groEL* a 65 kd Mr protein, *groES* a 15 kd Mr protein. In its native form *groEL* protein is a decatetramer, with its subunits arranged in a double ring with seven-fold symmetry (90).

Although *groEL* and *groES* are essential for cell growth at all temperatures

(C. Georgopoulos, personal communication), their role in cell growth is unclear. Temperature-sensitive *groEL* and *groES* mutants show an inhibition of both cellular DNA and RNA synthesis at the nonpermissive temperature (211, 228). Suppressors of one *groES* allele map to the *rpoA* gene that encodes a subunit of RNA polymerase (227). Furthermore, overproduction of GroEL and GroES suppress some *dnaA* alleles. *dnaA* is a gene essential for the initiation of *E. coli* DNA synthesis (66, 97). Since RNA polymerase is necessary for priming of DNA synthesis, these genetic results implicate the *groE* genes in some aspect of DNA replication.

GroEL and GroES are required for head assembly of lambda and T4 phages and for tail assembly of T5 phage. The effect of *groES*$^-$ mutations on lambda head assembly can be suppressed by mutations in *groEL* suggesting an interaction of the encoded proteins in vivo (210). Results of biochemical experiments suggest an association as well. The two proteins cosediment in a glycerol gradient in the presence of ATP and Mg^{2+}, and GroES binds to a GroEL-affinity column (34). Analysis of *groEL* mutants indicates that GroEL acts in lambda prophage head assembly at a step involving the bacteriophage-coded minor head protein B, in which B protein is oligomerized into a dodecameric structure (107). The B protein decamer is located on the head at the point of tail attachment.

Recently a heat inducible protein, (called hsp58), which is related to GroEL, has been identified in Tetrahymena (136, 137). hsp58 is constitutively expressed, and its level increases two-to-three-fold after heat shock. The majority of hsp58 is mitochondrial associated and in a nondenaturing gradient sediments as a 20–25S complex. Proteins that cross-react with an antibody directed against the Tetrahymena protein have been identified in a wide variety of species, including yeast, frogs, maize and human cells. Furthermore, a very abundant chloroplast protein called the Rubisco large subunit binding protein is related to GroEL (89). A large subunit of rubisco (ribulose biphosphate carboxylase-oxygenase) contains the catalytic site but is active only in oligometric combination with the small subunit. The GroEL-like protein is implicated in the assembly of the Rubisco multimetric complex. Similarities between this role in chloroplasts and the role in phage morphogenesis can be seen; both involve the assembly of multimetric complexes. The *groE* proteins may possibly play a similar role in DNA replication as well.

THE SMALL HSPS

The small hsps are a very diverse group. Different organisms have different numbers of small hsps, ranging from one, in *S. cerevisiae* (168), to upwards of 30 in higher plants (131). Great variance is also observed in molecular

weights, which range from 16 kd in the nematode *C. elegans* (182), to 40 kd in the protozoan *S. mansoni* (150). Even within species, considerable heterogeneity is observed (131). Nevertheless, the small hsps of different organisms are clearly related. They have similar hydropathy profiles (although such analyses must be treated with caution) and small regions of amino acid identity (See Figure 4). Their most invariant feature is the sequence aa1-aa2-glycine-aa3-leucine-aa4-aa5-aa6-aa7-proline-aa8, which is found near the carboxy terminus at a similar position in the hydropathy profiles of each protein. The amino acids designated aa1-8 are not invariant but are represented by only a small number of amino acids. For example, aa4 is threonine in most species; aa5 is always isoleucine, valine, or leucine. It is striking that the small hsps show much greater homology within organisms than between organisms. For example, members of a subgroup of the soybean small hsp family have 90% amino-acid identity with each other but only 20% amino-acid identity with the proteins of *D. melanogaster*, *C. elegans*, and *X. laevis* (148). Either the genes are subject to relatively frequent conversion events, or they have been frequently deleted and expanded during evolution. None of the well-characterized hsps of *E. coli* show significant homology to the small hsps of higher cells, and until recently, it was believed the protein family was eukaryotic in origin. However, the gene for an 18 kd antigen that shows homology with the eukaryotic small hsps has now been isolated from the mycobacterium *M. leprae* (247) (see Perspectives, above). It now appears that the small hsp family has existed for well over a billion years.

Though divergent in sequence, the small hsps are conserved in their structural properties. First, they share the property of forming highly polymeric structures, often called heat-shock granules, with sedimentation coefficients of 15–20S (9, 157, 191). As first reported for the fruit fly *D. melanogaster*, the small hsps have sequence homology with the vertebrate alpha-crystallins, which form similar structures (19, 96). Unfortunately, this characteristic has led to some confusion, as other particles of similar size, also composed of small molecular-weight proteins, are found in most eukaryotic cells (7, 188). These other particles, called prosomes, or proteasomes because of their involvement in proteolysis, have recently been distinguished from the heat-shock granules by three different research groups (8, 64, 157). The small hsps appear to share other physical properties as well. Both the mammalian proteins (9, 103) and the *Drosophila* (178) proteins are phosphorylated under a variety of different conditions. It is not yet known whether this modification is universal or serves any purpose. Finally, the small hsp particles isolated from *Drosophila*, sea urchin, and tomato cells (106, 157, 176) have been found to contain RNAs. The protein may serve to preserve translationally inactive messenger RNAs (157). However, this finding has been disputed (41), and mutations in the unique small hsp of yeast cells (see below) have no

affect on the stability of stored mRNAS in yeast spores (S. Kurtz, S. Lindquist, unpublished). Again, that this property is shared by such evolutionarily diverse organisms suggests it is significant, but its function is as yet unclear.

Finally, the small hsps share the property of being induced at specific stages in development at normal temperatures. These developmental induction patterns can be quite complex. In *D. melanogaster*, a total of seven small hsps

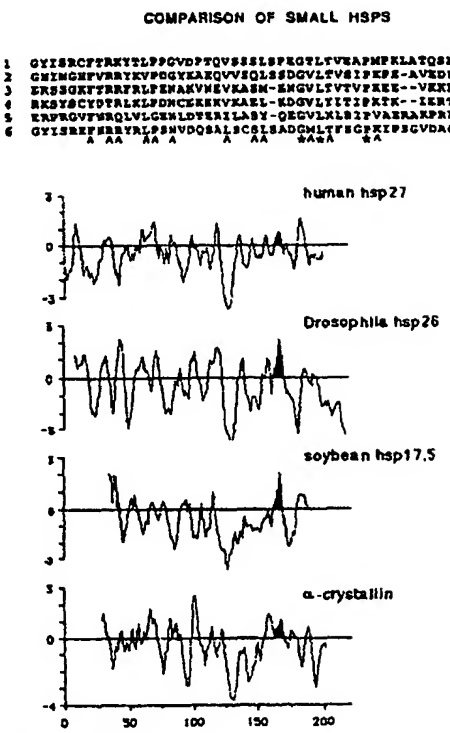

Figure 4 Comparison of the small hsps.
Top: the amino acid sequences of the most conserved domain, located at or near the carboxy terminus of several small hsps. 1. Human hsp27 (aa # 132–186) Hickey et al (91). 2. Drosophila hsp26 (aa # 127–180) Ingolia & Craig (96). 3. Soybean hsp17.5E (aa # 103–154) Czarnecka et al (50). 4. Pea chloroplast hsp21(aa # 183–232) Vierling et al (226). 5. Mycobacterium 18kd antigen (aa # 81–133) Nerland (151). 6. Bovine α-crystallin A chain (aa # 108–161) van der Ouderaa (217). (*) Invariant amino acids; (˙) identical amino acids in at least 4 of the 6 proteins. Bottom: hydropathy profiles of members of the small hsp protein family (as analyzed by the method of Kyte & Doolittle, (112), with a window of 6). The profiles are aligned by the most conserved domain, which is indicated in black.

have been identified, six of which clearly belong to the small hsp family (10, 96, 201). All of the genes are transcribed during the late larval and early pupal stages (10, 37, 198). *GENE 3* is expressed in the middle of embryonic development (163). *HSP 23* and *GENE1* are transcribed in young adults just after eclosion (10). *HSP27* and *HSP26* are expressed in nurse cells (together with *HSP83*) and are passed into developing oocytes (252). Detailed, tissue-specific analysis of one of these genes, *HSP26*, revealed further complexities, with expression concentrated in spermatocytes, nurse cells, epithelial tissues, imaginal discs, proventriculus, and neurocytes (78). At least some of this stage- and tissue-specific regulation is due to the hormone ecdysone (15, 208), which exerts its effect on transcription elements separate from the heat-shock elements on the small hsp genes. The elements that regulate transcription in spermatocytes can also be separated from those regulating transcription in nurse cells. These findings suggest that a highly complex regulatory system is operating on these genes.

Although less extensively characterized in other organisms, it seems likely that developmental induction of the small hsps will be universal. In *Saccharomyces* hsp26 is induced during stationary phase growth and during sporulation (111). In lilies several small hsps are induced during meiosis (R. Bouchard, personal communication). The presence of such developmental induction in these diverse organisms implies that small hsps play a role in development as well as in response to stress.

Unfortunately, the molecular role of these proteins is at present a complete mystery. Several studies have suggested that the small hsps are responsible for acquired thermal tolerance. For example, in *D. melanogaster*, ecdysone induces both thermotolerance and the small hsps, but not hsp70 (15). Also, thermosensitive tomato cells constitutively synthesize hsp68 and hsp70, but they become thermotolerant when given a mild heat treatment that induces the small hsps (156). A possible problem with these studies is that small changes in other proteins might have important biological consequences and yet escape detection. Two lines of genetic analysis also support this view. First, a mutant strain of *Dictyostelium* that fails to synthesize the small hsps also fails to acquire thermotolerance (129). However, since the primary lesion in this mutation has not been identified, it may be the failure to acquire thermotolerance that is, in turn, responsible for the failure to synthesize the small hsps. More recently, variants of a CHO cell line, selected for heat-resistance, were found to have elevated levels of hsp28, and only hsp28 (115).

On the other hand, in *Drosophila*, deletion (197) and insertion mutations in individual small hsp genes (60) as well as pseudo-mutations induced by antisense-RNA (135), have no noticeable effects on viability. Of course, in *Drosophila* cells other members of the small hsp family might cover the function of the mutated protein. The genetic analysis in *S. cerevisiae* is more

compelling. In this organism there is only one major small hsp. Deletion mutations have no detectable effect on (*a*) respiratory or fermentative growth at 25°, 35°, 37° or 39°C, the latter being the highest temperature at which these cells will grow, (*b*) basal or acquired thermotolerance in respiring or fermenting cells, (*c*) spore development with or without a heat shock, (*d*) spore germination with or without a heat shock, (*e*) spore viability during long-term storage, (*f*) dessication tolerance, (*g*) ethanol tolerance, (168), or (*h*) growth under anaerobic conditions at high or low temperatures (M. Schlessinger, N. Collier, personal communication). The assays used in these experiments were very sensitive. Large differences in thermotolerance and growth rates in different media and at different temperatures were found in comparing various common laboratory strains of yeast. But, in comparing isogenic wild-type and *hsp26*$^-$ cells, in either high thermotolerance or low thermotolerance genetic backgrounds, no differences were observed. Taking an alternative approach, *hsp26* coding sequences were placed under the control of the *gal1* promoter, to provide induction of the protein prior to heat shock. Cells carrying the wild type *HSP26* gene, the *hsp26* deletion mutation, or *pGAL1-HSP26* showed no significant differences in basal or acquired thermotolerance when grown on galactose (A. Taulien, A. Petko, S. L. Lindquist, unpublished).

Three explanations for the yeast results seem plausible. First, it may be that the function of hsp26 is covered by another protein in *Saccharomyces*. If so, that protein bears little homology to hsp26. No cross-reacting genes have been detected by low stringency DNA hybridization, and no cross-reacting proteins have been observed with a polyclonal anti-hsp26 serum (168; see below). Given the divergence observed in this protein family, other members of the family might well escape detection. However, if other members of the family are present, they must not be strongly heat-inducible, or they would have been detected on gels. A second possibility is that the function of the protein may be much more subtle than previously expected. Genes that give very small advantages for survival under very particular circumstances can still be selected for in evolution. Finally, however unpalatable the notion, it is possible that the small hsp genes represent an ancient form of selfish DNA.

Investigations of the intracellular location of the small hsps have not helped to clarify their functions. Several cell-fractionation studies have localized them to chromatin, nucleoli, and the nuclear skeleton. More recently, immunofluorescence and immunoelectron microscopy has demonstrated that they are in large, cytoplasmic, granular aggregates, often in close proximity to the nucleus (41, 152). During heat shock they partially localize to the nucleus, although this has been disputed (reviewed in 180). In soybeans, at least one member of the small hsp family is transported to chloroplasts and carries a signal sequence that is clipped during transport (226). In the yeast *S.*

cerevisiae, immunofluorescent staining has revealed that the intracellular location of hsp26 has a complex but as yet unilluminating dependence on the physiological state of the cell (180).

At present, the small hsps are the most baffling of all the hsps. They are abundant, ubiquitously distributed proteins induced both by heat shock and by normal developmental cues. They are a remarkably diverse group, conserved more in their structural properties than in their amino-acid sequences. In fact, were it not for their common induction patterns, the homologies in their amino acid sequences would not be sufficient to suggest a common function. Some evidence suggests they are important in thermotolerance; genetic analysis in yeast suggests they play no role in this phenomenon.

UBIQUITIN

Ubiquitin, a highly conserved 76-residue protein, which as its name implies is found in all eucaryotic cells, is induced by heat (21, 22, 72). In all five eucaryotic species examined, it is synthesized as a polyprotein termed polyubiquitin, generally consisting of tandem repeats of the protein coding sequences with no spacer regions. Ubiquitin is found in cells either free or linked via its terminal glycine residue to a variety of cellular proteins. The conjugation process, which is ATP dependent, is apparently an essential precondition for selective degradation of intracellular proteins.

The first mutation identified in a gene involved in the ubiquitination process was in the strain ts85, a temperature-sensitive derivative of a cell line established from a spontaneous mouse mammary carcinoma. This cell cycle mutant has a temperature sensitive defect in the ubiquitin activating enzyme E1 (71). At the nonpermissive temperature (which is below the temperature necessary to induce hsps in mammalian cells) synthesis of ubiquitin and some members of the hsp70 family is increased. This result led to the suggestion that inactivation or overloading of the ubiquitin system leads to the induction of heat-shock proteins (71). It has also been hypothesized that the common induction signal in the heat-shock response is the denaturation of proteins (3). Inducers such as amino acid analogs, heat, and ethanol might cause a fraction of the protein of the cell to denature. The heat shock–specific transcription factor is present predominantly in an inactive form in noninduced cells. The hypothesis is based on the assumption that at any time some of the factor is converted to an active form and that this active form is labile, rapidly inactivated proteolytically, and thus does not accumulate in noninduced cells. When cells are stressed, the protein that is denatured could compete effectively with the active form of the transcription factor for the limited proteolytic machinery, thus resulting in the factor's stabilization. The induction of hsps in line ts85 is consistent with this model. Also, it is known that proteins containing amino acid analogs are degraded by the ubiquitin system, and that amino acid analogs induce the heat-shock response. However, if this model is correct it is difficult to understand the induction of hsps in situations in which the temperature is raised to only 37°C (a condition which will induce the response in yeast), or to even lower temperatures, as is the case with arctic fishes. It had been proposed that ubiquitin is directly involved in the modification of the heat shock transcription factor and therefore the induction of the response (145). However, no evidence supports this idea. Since it is now known that phosphorylation of the transcription factor occurs upon heat shock, it is believed that this modification is responsible for activation (199).

Ubiquitin genes have been isolated from several organisms; the yeast genes have been studied most extensively. The four yeast genes all encode hybrid proteins in which ubiquitin is fused at its carboxyl end either to itself, as in polyubiquitin encoded by *UBI4* (160), or to unrelated amino acid sequences as in the case in *UBI1*, *UBI2* and *UBI3* (159). Of these four genes only *UBI4* is heat inducible. A precise deletion of *UBI4* has been constructed and substituted into the genome (72). The *ubi4* deletion grows at rates comparable to wild-type strains at least between 23 and 36°C. The steady-state levels of free ubiquitin are very similar to those in wild-type strains, indicating that free ubiquitin is being generated from the products of the other genes. A number of phenotypes of the *ubi4* strains have been observed. *ubi4* strains are hypersensitive to exposure to 38.5°C, a borderline growth temperature for yeast. While about 60% of wild-type cells maintain colony-forming ability for 16 hours at 38.5°, only 1–5% of mutant cells survive. The *ubi4* strains are also more sensitive than wild-type strains to amino acid analogs and starvation for nitrogen and carbon. *UBI4* is also required for maintenance of spore viability. Since ubiquitin is thought to be necessary for the degradation of abnormal proteins, there is probably an increased demand for ubiquitin during times of stress, such as after a heat shock or in the presence of amino acid analogs. Although in a *ubi4* mutant there is sufficient free ubiquitin generated from the products of the *UBI1*, *UBI2* and *UBI3* genes under normal growth conditions, during or after stress there is not; thus, a requirement for *UBI4* under those conditions.

OTHER PROTEINS

Eukaryotes

In various cell types, many other proteins have been found to be heat-inducible. These proteins may be produced in lesser quantities than the classic hsps, may be less strongly heat-inducible, or may be of more limited phylogenetic distribution. They have been little studied and the elucidation of their functions is just beginning.

High-resolution 2-D gel electrophoresis of various species has identified a large number of heat-inducible proteins, which are among the less abundant cellular proteins. One of these, whose rate of synthesis may be induced up to 15-fold, was identified as the α-subunit of eukaryotic initiation factor 2 (eIF-2α). The phosphorylation of eIF-2α was previously proposed as the mechanism for translational repression of normal proteins in heat-shocked mammalian cells (57). Its induction by heat shock might then play a role in restoring normal levels of translation after heat shock. However, the role of eIF-2α in regulating heat-shock translation is still controversial (132), and no data on the generality of its induction by heat are yet available.

The gene for a 35-kd heat-inducible protein in *S. cerevisiae* was identified as one of three closely related genes in the yeast genome (169), previously identified as the glyceraldehyde-3-phosphate dehydrogenase gene family (G3PDH). The isoform induced by heat is also one of the most abundant cellular proteins at normal temperatures. A strain carrying a disruption mutation grows more slowly than the wild-type in rich media at 25 and 37°C, but it grows at the same rate as the wild-type in rich media at 39°C and in minimal media at 25°C. Apparently the heat-inducible G3PDH isoform facilitates rapid growth over a broad range of temperatures. However, when growth rates are reduced, either by high temperatures or by a lack of nutrients, it is dispensable. In media in which the mutant and wild type grow at the same rate, the mutant is more resistant than the wild-type to killing at 50°.

A heat-inducible protein of ~35-kd has been reported in many cell types and may be a common feature of the response in eukaryotes. In most cases the identity of the protein is unknown but in *Xenopus* embryos hsp35 has also been identified as G3PDH (153). It seems likely that its role in the response may relate to the fact that ATP levels are reduced by heat shock (69) while respiration is adversely affected. G3PDH may be induced to help restore ATP concentrations to normal by increasing the rate of glycolysis. That two other glycolytic enzymes, enolase (94) and phospho-glycerate kinase (172), are induced by heat shock lends support to the hypothesis. The induction of enolase (hsp48) requires further note. It was originally reported that yeast cells carrying mutations in the enolase gene are thermosensitive. However, further study of isogenic strains revealed no difference in thermotolerance between *eno1* and *ENO1* strains (H. Iida, personal communication).

Other proteins may be abundant and strongly heat-inducible but idiosyncratic in their appearance, restricted to certain organisms, to certain cell types within organisms, or to certain developmental stages. An example of this type is a collagen-binding protein of chicken embryos (183). In the liver this 47-kd glycoprotein is present in fibrocytes, Kupffer cells and smooth muscle, but it is absent from hepatocytes, bile duct epithelia and sinusoidal endothelium. Other example are (*a*) a 180-kd protein in endothelial cells identified as thrombospondin (102), (*b*) several cuticle-like proteins in epidermal cells of *Manduca*), (*c*) 28 proteins in the male accessory glands of *Sarcophaga*, (100) and (*d*) six heat-inducible proteins produced by fruiting cells but not vegetative cells of *Myxococcus xanthus* (259). This list, though incomplete, is thought provoking. Specialized cells may induce special proteins, due to the particular pathological effects that heat produces in them.

Prokaryotes

Seventeen heat-shock proteins have been identified in *E. coli*. Ten of these are the products of known genes (see F. Neidhardt, R. VanBogelen, 149, for a review). Because of their structural relationship to eucaryotic heat shock genes, three genes, *dnaK*, *groEL* and *htpG*, have been discussed above. Two others, *grpE* and *lon*, are discussed below because of their relationship to *dnaK* and the involvement of proteolysis with the heat-shock response, respectively.

grpE encodes the 24 kd hsp, B25.3 (5). Like DnaK and DnaJ proteins, GrpE is necessary for lambda phage growth at all temperatures, although its role is not clear. Analysis of temperature sensitive mutants indicate that GrpE protein is also essential for cell growth at least at high temperatures (43.5°C and above) (5). The *dnak* and *grpE* proteins interact, as shown both by sedimentation in glycerol gradients and by binding of *GrpE* to a DnaK affinity column. The proteins dissociate in the presence of ATP (253). Extragenic suppressors of the *grpE* 280 mutation have been mapped to *dnaK*, supporting the existence of functional interactions between GrpE and DnaK in vivo (C. Georgopoulos, personal communication).

The ATP-dependent Lon protease is a heat-inducible protein of 94 kd. Mutations in the *lon* gene cause a 2–4-fold reduction in the rate of degradation of incomplete peptides or proteins containing amino acid analogs (39). Also, *lon* mutants have a decreased rate of breakdown of short-lived regulatory proteins, causing a variety of phenotypes (142), including the accumulation of large quantities of mucopolysaccharides, an abnormal SOS response and a decreased ability to lysogenize phage lambda. Overproduction of Lon results in increased rates of degradation of abnormal proteins and normal cellular proteins. The growth of cells containing greater than normal amounts of Lon is also impaired (80). Therefore *Lon* is thought to play a major role in the degradation of abnormal proteins and in regulating the turnover of normal proteins. The fact that both eucaryotic and procaryotic cells have at least one heat-shock protein (the ubiquitin and Lon proteins, respectively) that is involved in proteolysis suggests that one role of the response is the destruction of abnormal proteins accumulated during the stress.

Thermotolerance

The heat-shock proteins are postulated to protect organisms from the toxic effects of heat and other forms of stress. The many mechanisms employed to

ensure that hsps are produced as rapidly as possible after temperature elevation, and the relationship between the temperatures that will induce the proteins in various organisms and the temperature fluctuations of their environment—both support this view. More compellingly, in a remarkable range of cells and organisms, incubation at temperatures that induce the hsps produces tolerance to much more extreme temperatures (122, 148). Examples include vertebrate tissue-culture cells, whole mice, *Drosophila* embryos, larvae, pupae and adults, slime molds, sea urchin embryos and plutei, soybean seedlings, yeast, and bacterial cells. The protective effects of pretreatment are not only manifest in lethality. Sublethal heat treatments induce developmental anomalies in many organisms, including vertebrates, insects, and plants, and preheat treatments reduce or eliminate these defects (141). Of course, many protective changes in physiology might be made during the pretreatment. Is the induction of hsps a critical factor? Here we review experiments that examine the hypothesis. The results of most support it; some do not.

The Correlation Between Hsp Synthesis and Thermotolerance

In experiments measuring the rate of thermotolerance development, it closely parallels the rate of hsp acumulation. Moreover, the decay of thermotolerance when cells are returned to normal temperature, parallels the degradation of hsps. Tolerance can also be induced by other types of conditioning treatments. These have in common the property of inducing hsps. Exposure to ethanol, hypoxia, and heavy metal ions are commonly employed. Such treatments do not induce heat-shock proteins in all organisms, but when they do, they also induce thermotolerance. Significantly, the converse is also true. That is, heat treatments induce tolerance to ethanol, anoxia, and several other forms of stress, underscoring the broadly protective nature of the response. A notable exception is the induction of hsps by amino-acid analogs, which is not accompanied by the induction of thermotolerance. Since proteins that have incorporated amino acid analogs are not likely to be functional, this tends to support, rather than negate, the hypothesis. (Reviewed in 32, 79, 122, 127, 141, 148, 205)

Early in the embryonic development of many organisms, including fruit flies, sea urchins, frogs, and mammals, hsps are not inducible and the organism is hypersensitive to thermal killing. At the time when hsps become inducible, the organism becomes more thermotolerant (reviewed in 25). Sperm development is extremely sensitive to high temperatures. In *Drosophila*, primary spermatocytes do not respond to high temperatures by inducing hsp70 (257). In rodent brains, different cell types exhibit marked differences in hsp70 induction and these correlate with the ability of individual cells to survive ischemia and heat shock (221). In other experiments the induction of

hsps has been blocked by inhibitors such as cycloheximide. Usually, the acquisition of thermotolerance is also blocked (127, 134).

There are, however, several counter-examples. In the mouse, spermatocytes do respond to heat by producing hsp70 (2). And a block in protein synthesis in yeast cells does not prevent the induction of thermotolerance by mild heat pretreatments (235). Since yeast cells produce hsps at moderate levels even at normal temperatures, the pretreatment may provide an opportunity to activate pre-existing proteins. Similar results have been obtained in some mammalian cell cultures, and the same argument may apply (120).

Mutations That Alter Hsp Synthesis

In *E. coli*, a mutation originally characterized as temperature sensitive for growth is now known to be an *amber* mutation in the *rpoH* gene, which produces the sigma factor required for expression of hsps (149, 245). When various suppressors are introduced into these cells, their ability to grow at high temperatures correlates with the efficiency of the suppressor. Furthermore, when wild-type strains are transferred from 30°C to 42°C, they acquire tolerance to 55°C, but when mutant strains carrying a ts *amber* suppressor are exposed to 42°C, they do not. Temperature resistant variants isolated from such strains are partially or fully restored for hsp synthesis (213).

In another series of experiments (219), the coding sequences of the *rpoH* gene were placed under the control of other promoters, allowing artificial induction of hsps at normal temperatures with IPTG. IPTG-induced cells showed no increase in thermotolerance, compared to uninduced cells, when they were exposed directly to high temperatures. As with wild-type, they required a conditioning treatment at 42°C, and this treatment was ineffective in the presence of chloramphenicol. A cautionary note is that hsps were not found in the same relative concentrations after IPTG induction as after heat shock, and a few hsps were not induced at all.

It has not been possible to expose eukaryotes to the same tests. The gene encoding the heat-shock transcription factor is essential in yeast (G. Weiderech, H. Pelham, personal communication), and conditional mutations have not yet been produced. However, other yeast mutations do support the argument that hsps are involved in thermotolerance. As discussed above, strains carrying mutations in the *SSA1* and *SSA2* genes, members of the *HSP70* gene family, constitutively overexpress other hsps. When these cells are directly exposed to high temperatures, they are nearly as thermotolerant as the wild-type strain is after a conditioning pre-heat treatment (45). Strains carrying mutations in *HSP83* and *HSC83* show reduced thermotolerance when grown in a medium that supports respiration, but not when grown in media that support fermentation (K. Borkovich, F. Farrell, D. Finkelstein, S. Lindquist, manuscript in preparation). Ubiquitin mutants are hypersensitive to

chronic heat stress, that is, incubation at temperatures that are just slightly above their maximum growth temperature (72). A yeast mutant, *hsr1 (cyr1)*, selected for thermoresistance synthesizes two 48 kd hsps (enolase isoforms) and two other proteins of 73 and 56 kd (94). Finally, yeast cells carrying mutations in various genes that regulate cAMP metabolism fail to respond to nutrient deprivation and are much more sensitive to heat than are wild-type cells (30). This may be because nutrient deprivation normally induces hsp accumulation in wild-type cells.

These experiments provide other important information. First, it is clear that the factors which permit cells to grow at the upper end of their natural temperature range differ from those which permit them to survive short exposure to extreme temperatures. The *ssa1 ssa2* mutant, which has increased tolerance at 50°C, is temperature sensitive for growth (45). The *hsp83* and *hsc83* mutants are also ts for growth, yet have normal basal and acquired thermotolerance when growing by fermentation (K. Borkavich, F. Farrell, D. Finkelstein, S. Lindquist, manuscript in preparation). The latter mutations also suggest that different proteins may be required under different growth conditions, since they have reduced thermotolerance when growing by respiration. The ubiquitin mutations separate the mechanisms required for surviving chronic exposure to superoptimal temperatures (under these conditions they have reduced levels of survival), from those required for surviving short exposure to extreme temperatures (they have normal survival) (72; D. Finley, personal communication). The *hsp35* mutations separate effects on growth rate from thermotolerance. In media in which they grow as well as do wild type, they have higher than normal levels of thermotolerance. In media in which they grow more slowly than wild type, they have normal levels of thermotolerance (169).

Experiments in vertebrate cells complicate the picture further. The results of many experiments implicate hsp70 in thermotolerance, since thermotolerance shows the best correlation with hsp70 concentrations (122). In cells subjected to repeated lethal heat treatments, in order to select thermoresistant variants, the only protein that is constitutively overproduced is hsp70 (122). However, another thermoresistant cell line constitutively overproduces only hsp90 (244), another overproduces only hsp28 (J. Landry, personal communication), and yet another overexpresses hsp89 and hsp68 and a novel hsp70 variant (4). In a complementary series of experiments, cells that have reduced abilities to survive high temperatures are defective in induction of hsp70. On the other hand, some cell lines with widely different levels of thermotolerance exhibit no qualitative or quantitative differences in hsp synthesis (67). Also, mouse cells transformed with SV40 show increased sensitivity to heat, yet both the constitutive and inducible levels of hsps are higher than in the parental line (258).

Overall, a great many experiments support the hypothesis that hsps are important components in the induction of thermotolerance. It seems almost certain that they are. However, in several cases, the synthesis of hsps is not sufficient to provide thermotolerance, and in others, it does not appear to be necessary. There are too many contrary reports, in a variety of different systems, for them to be dismissed. Accepting the premise that hsps do play a role in thermotolerance, two other conclusions seem appropriate: First, cells must have a variety of mechanisms for coping with the toxic effects of high temperatures that are separate and apart from the synthesis of hsps. If the state of metabolism, differentiation, or experimental intervention should prevent these from being activated, the synthesis of hsps might be irrelevant. Second, cells may be killed by different lesions when they are in different states of metabolism or in different stages of differentiation, as when they are exposed to extreme temperatures for short periods or moderately high temperatures for long periods. Different hsps may protect cells from different lethal lesions. Although many of the toxic effects of heat have been defined—reductions in protein synthesis, transcription, and RNA processing, rearrangements of the cytoskeleton, changes in membrane permeability, disruptions in oxidative respiration, photosynthesis, etc.—it is still not clear which of these are critical lethal lesions (reviewed in 181). Defining the specific lethal lesions that are induced by high temperatures would be of great help in defining the mechanisms that are employed for protection. The very nature of the heat-shock response suggests it is homeostatic. The extraordinary degree of conservation observed in most of the proteins indicates the underlying basis for the protective strategies is universal.

REGULATION OF THE RESPONSE

It is difficult to close this review without briefly considering regulation, both because regulation of the proteins is closely associated with regulation of thermotolerance and because studies of heat shock regulation have provided many important insights on the control of gene expression. In *E coli*, the heat-shock response is transcriptionally regulated by the cellular concentration of σ^{32}, a sigma factor that binds to core RNA polymerase and redirects it to heat-shock promoters (82, 114, 245). Simple as it would appear, the regulation of σ^{32} concentrations is itself complex. Transcriptional, translational, and posttranslational mechanisms are all employed. The σ^{32} gene, *rpoH*, is transcribed constitutively by the standard, σ^{70}-containing polymerase. An extremely rapid response to heat is achieved by an immediate increase in the translational efficiency of the σ^{32} message, by an increase in the concentration of the σ^{32} message, and by the stabilization of the normally very unstable protein product (203, 209).

Transcriptional regulation also plays an important role in regulation in eukaryotes. Here the essential transcription factor, HSF (for Heat-Shock Factor), preexists in sufficient concentration but is inactive in form. The factor is rapidly activated in response to temperature elevation by posttranslational modification, producing an extremely rapid increase in heat-shock transcription (199, 243). In higher eukaryotes, additional mechanisms are employed to circumvent other barriers to a rapid response. For example, in organisms with larger genomes and a hierarchy of chromatin structure, the heat-shock genes are preset in an open chromatin configuration at normal temperatures, with hypersensitive sites at their 5' ends (101, 242). In at least some cases, polymerase is already engaged on these genes but is blocked by a negative regulatory mechanism that is immediately released when the temperature is raised (77). Translational mechanisms also play a vital role. While heat-shock messages are translated with high efficiency, preexisting messenger RNAs are translationally repressed, reducing the competition for translation (126, 202). At the same time, hsp70 messenger RNAs, which are extremely unstable at normal temperatures, are stabilized by heat shock (167, 207).

Thus, in both prokarotes and eukaryotes many regulatory mechanisms, acting transcriptionally and post-transcriptionally, are employed to induce the proteins, their unifying theme being to ensure that the proteins are induced as rapidly as possible.

CONCLUDING REMARKS

A theme that runs throughout this review is the involvement of hsps in protein-protein interactions. Small hsps form large aggregates; hsp90 interacts with steroid receptors and with the virus encoded transforming protein, *src;* hsp70 and related proteins with clathrin baskets, DNA replication complexes, ER proteins, and the cellular tumor antigen p53. Much of this data is at least consistent with the notion that some hsps are involved in protein folding and assembly (or disassembly) of protein complexes. The heat inducible proteins may be involved in reassembling structures damaged by heat shock or other stresses.

This review focuses on the role of hsps and related proteins in normal growth; the work discussed demonstrates a vital role for at least some of these proteins. The question whether the heat-inducible proteins perform the same function as the constitutively expressed proteins or carry out specialized functions remains unanswered. It is possible that the heat inducibility of some members of multigene families has evolved merely to increase the amount of total protein. However, it is reasonable to suppose that those proteins whose expression increases after stress, although perhaps able to perform some of the same functions as their noninducible relatives, have evolved the capacity to specifically cope with the physiological stresses rendered by heat and other

insults. Hopefully, over the next few years, functional distinctions between heat inducible and constitutive proteins and their roles in thermotolerance will be elucidated.

The diverse processes in which hsps function have been implicated indicates that these proteins are involved in many cellular processes. The job ahead for workers in the field is to determine which of these biochemical interactions observed in vitro, actually occur in vivo and which are essential for normal cell growth. The powerful approaches of both genetics and biochemistry will be needed to answer these questions.

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